



Published in final edited form as:

*Mol Aspects Med.* 2015 December ; 46: 78–84. doi:10.1016/j.mam.2015.08.003.

## Glycogen Phosphorylation and Lafora disease

**Peter J. Roach**

Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, 635 Barnhill Drive, Indiana University School of Medicine, IN 46202

### Abstract

Covalent phosphorylation of glycogen, first described 35 years ago, was put on firm ground through the work of the Whelan laboratory in the 1990s. But glycogen phosphorylation lay fallow until interest was rekindled in the mid 2000s by the finding that it could be removed by a glycogen-binding phosphatase, laforin, and that mutations in laforin cause a fatal teenage-onset epilepsy, called Lafora disease. Glycogen phosphorylation is due to phosphomonoesters at C2, C3 and C6 of glucose residues. Phosphate is rare, ranging from 1:500 - 1:5000 phosphates/glucose depending on the glycogen source. The mechanisms of glycogen phosphorylation remain under investigation but one hypothesis to explain C2 and perhaps C3 phosphate is that it results from a rare side reaction of the normal synthetic enzyme glycogen synthase. Lafora disease is likely caused by over-accumulation of abnormal glycogen in insoluble deposits termed Lafora bodies in neurons. The abnormality in the glycogen correlates with elevated phosphorylation (at C2, C3 and C6), reduced branching, insolubility and an enhanced tendency to aggregate and become insoluble. Hyperphosphorylation of glycogen is emerging as an important feature of this deadly childhood disease

### Keywords

glycogen; phosphorylation; Lafora disease; laforin; malin

## 1. Introduction

Bill Whelan's career has spanned many decades and many research interests, though the latter were unified by his passion for carbohydrates, and storage polymers like glycogen and starch in particular. My own work since the 1980s has sometimes overlapped with Bill's, especially in the area of glycogenin, whose discovery ranks among Bill's most notable achievements and which is addressed in other articles of this issue. Here, though, I will focus on another, seemingly esoteric feature of glycogen metabolism on which Bill had made important observations in the early 1990s, namely the covalent phosphorylation of glycogen. His work lay relatively undisturbed in the literature until quite recently when it began to be appreciated that excessive phosphorylation of glycogen was associated with, and

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

might even cause, Lafora disease, a rare but deadly teenage-onset form of epilepsy. And so our work once again bumped into key findings made by the Whelan laboratory.

## 2. Glycogen phosphorylation

### 2.1. History

Phosphate is a relatively abundant and ubiquitous biomolecule and, as such, is a potential and frequent contaminant of many purified cellular constituents. Such was true for purified glycogen and, indeed, in early studies, low phosphate content was viewed as a positive indicator of the purity of the glycogen (Mordoh et al., 1966; Northcote, 1954; Wanson and Drochmans, 1968). Already, however, plant starch, which resembles glycogen in being a branched polymer of glucose, was recognized as containing covalent phosphate (Hizukuri et al., 1970). The first convincing report of stably bound phosphate in glycogen was by Fontana (Fontana, 1980) in 1980. Whelan and colleagues followed up with a series of studies (Kirkman and Whelan, 1990; Lomako et al., 1994; Lomako et al., 1993) in which they confirmed the presence of covalent phosphate, suggested that it was present as a C6 phosphomonoester or as a C1-C6 phosphodiester, and proposed the existence of an enzyme, separate from glycogen synthase, that could transfer a glucose-phosphate moiety from UDP-glucose to form the phosphodiester linkage.

### 2.2. Chemistry

The chemistry of the attachment of phosphate to glycogen was re-visited recently, in large part because of the discovery of a mechanism to release it from glycogen by a phosphatase called laforin (Tagliabracci et al., 2007). Laforin is the product of the *EPM2A* gene, one of two genes whose mutation causes Lafora disease (Chan et al., 2003; Minassian et al., 1998; Serratos et al., 1999). In *Epm2a*<sup>−/−</sup> mice (hereafter designated laforin<sup>−/−</sup> mice), a rodent model of the human disease, the phosphorylation of glycogen increases up to ten-fold as the animals age (Tagliabracci et al., 2007) and the excess phosphate appears to contribute significantly to the pathology of the disease (see section 2.5). The stoichiometry of glycogen phosphorylation is low, in the range of 1 phosphate per 500 glucoses to 1 phosphate per several thousand glucoses, depending on the source of glycogen and the particular study. Because of the scarcity of the phosphate, we hydrolyzed rabbit muscle glycogen with glucosidases and enriched for negatively charged species by anion exchange chromatography (Tagliabracci et al., 2011). Analysis of the bound material by mass spectrometry indicated a mixture of compounds dominated by species with masses corresponding to hexose oligosaccharides plus a phosphate, consistent with the purification of phospho-oligosaccharides of glucose. Despite the size-heterogeneity, analysis of this sample by NMR gave clear signals indicative of glucose phospho-monoesters at the C2 and the C3 atoms but did not observe either C6 phosphate or phosphodiesters. A subsequent study (Nitschke et al., 2013), using similar methods for purification of phospho-oligosaccharides, detected C2, C3 as well as C6 phosphate by NMR, but, as in our work, did not observe phospho-diesters (Tagliabracci et al., 2011). In addition, Nitschke et al. (Nitschke et al., 2013) measured glucose-6-P directly in hydrolysates of glycogen from mouse and rabbit sources. The two papers presented conflicting views as to the presence of C6 phosphate in glycogen; we therefore pursued the question further, testing the hypothesis

that methods of purifying glycogen might affect the phosphorylation pattern (DePaoli-Roach et al., 2015). We analyzed rabbit muscle glycogen isolated by our previous, relatively gentle procedure, in fact following more or less the old Whelan protocol used to preserve glycogenin attached to the glycogen (Kennedy et al., 1985). In addition, we used a more extreme procedure in which powdered frozen muscle is treated first with boiling KOH. Results from either protocol were similar and in both cases we now detected C6 phosphorylation by NMR analyses (DePaoli-Roach et al., 2015). We also developed a sensitive assay for glucose-6-P in glycogen hydrolysates and were able to quantitate C6 phosphorylation in glycogen samples that, combined with analysis of total covalent phosphate, permitted us to calculate the relative contribution of C6 and C2 + C3 phosphorylation to the total phosphate content. C6 phosphate typically making up around 20% of the total phosphate with C2 + C3 phosphate accounting for the majority, around 80%, in either mouse or rabbit muscle glycogen samples (DePaoli-Roach et al., 2015). This phosphate distribution contrasts with amylopectin in which most phosphate is associated with C6 (Blennow et al., 2002; Stitt and Zeeman, 2012), for example ~90% in our recent study of potato amylopectin, the rest of the phosphate at C3, and little evidence for significant phosphorylation at C2 (DePaoli-Roach et al., 2015). Though the presence of phosphodiesterases cannot be completely discounted, these recent studies did not find any indications for their existence.

### 2.3 Metabolism

The metabolism of the covalent phosphate within glycogen is not yet fully understood. The laforin phosphatase (Minassian et al., 1998; Serratos et al., 1999) by sequence belongs to the atypical dual specificity protein phosphatase (DSP) sub-family (Alonso et al., 2004). Laforin is unique in being the only protein phosphatase in the genome that contains a built-in carbohydrate binding domain (CBM20) at its N-terminus as well as a phosphatase domain. After an essentially fruitless search for protein substrates, it emerged that laforin can act on polysaccharide substrates like amylopectin (Tagliabracci et al., 2007; Worby et al., 2006) and glycogen (Tagliabracci et al., 2007). In biochemical experiments, mutation of the CBM to disable carbohydrate binding also eliminates the ability of laforin to dephosphorylate glycogen while leaving the active site capable of hydrolyzing generic substrates like *p*-nitrophenol phosphate (Tagliabracci et al., 2007). Glycogen purified from the muscle of mice lacking laforin contains elevated levels of phosphate compared with controls (Tagliabracci et al., 2008; Tagliabracci et al., 2007). Therefore, it is currently quite well accepted that laforin functions as a glycogen phosphatase *in vivo*. Plants contain two glycan phosphatases, SEX4 and LSF2, that remove the phosphate esters present in amylopectin (Fettke et al., 2009; Stitt and Zeeman, 2012; Zeeman et al., 2010). These enzymes resemble laforin in that they also contain CBMs although the architecture is different, the CBMs being C-terminal. In addition, the plant phosphatases are thought to act physiologically on smaller glucans generated from the breakdown of amylopectin (Fettke et al., 2009; Stitt and Zeeman, 2012; Zeeman et al., 2010). Although laforin can dephosphorylate phospho-oligosaccharides larger than three glucoses in length, it most likely acts on large glycogen molecules *in vivo*.

Glycogen is also degraded in lysosomes and, though the relative contribution of this process compared with the classic cytosolic pathway (phosphorylase/debranching enzyme) is not well defined, its importance is underscored by the severity of Pompe disease, in which the lysosomal  $\alpha$ -glucosidase is defective (Hirschhorn and Reuser, 2000; Raben et al., 2002; Reuser et al., 1995). Presumably the battery of hydrolytic activities within the lysosome would normally have no difficulty removing any phosphate attached to glucose or oligosaccharides derived from glycogen. The specifics of the trafficking of glycogen to the lysosome are poorly understood though it is likely to have similarities to autophagic pathways and to involve vesicular engulfment of glycogen particles. In a mouse model of Pompe disease, glycogen-laden autophagosomes are seen to accumulate in muscle (Fukuda et al., 2006). We have proposed that the glycogen binding protein Stbd1 might play a role in mediating the vesicular transport of glycogen to lysosomes (Jiang et al., 2010). Furthermore, since Stbd1 binds more tightly to the hyperphosphorylated glycogen purified from laforin-/- mice, one could even imagine the selective disposal of excessively phosphorylated and abnormal glycogen. This idea interestingly aligns with suggestions of Bill Whelan's from over twenty years ago (Lomako et al., 1994) that glycogen phosphate might have something to do with lysosomal targeting and that phosphorylation of glycogen molecules increases with their age. However, the idea that glycogen phosphorylation is related to lysosomal targeting remains a hypothesis at this time.

The mechanism by which phosphate is introduced into glycogen remains unresolved (Table 1). From the polysaccharide literature, there were two main possibilities. The first was the glucose-1-phosphotransferase proposed by Lomako et al. (Lomako et al., 1993) which would bring phosphate in a C1-C6 phosphodiester linkage to form a novel type of branch point in glycogen. The second would be a glycogen water dikinase by analogy to the enzymes that are responsible for the phosphorylation of amylopectin. Glucan water dikinase (GWD) introduces phosphate at C6 (Mikkelsen et al., 2004) and a second enzyme, phosphoglucan water dikinase (PWD), phosphorylates C3 but preferentially in substrates that have been previously phosphorylated at C6 (Baunsgaard et al., 2005; Kotting et al., 2005). However, neither bioinformatic nor biochemical investigations have yielded evidence for comparable enzymes in mammals. A priori, one could also postulate the existence of glycogen kinases that would transfer the  $\gamma$ -phosphate of ATP, or other nucleoside triphosphates, to a glucose residue within glycogen. Alternatively, other high energy compounds, such as phospho-*enol*-pyruvate or creatine phosphate, could serve as phosphate donors. Once introduced, phosphomonoesters in glucose residues might be re-distributed among glucose carbons in reactions somewhat akin to the phosphoglucomutase of intermediary metabolism. If such an enzyme existed, we might need only one primary mechanism for the introduction of phosphate into the polysaccharide. Phosphate migration is a well-established phenomenon in the chemistry of sugar esters (MacDonald, 1972; Patel and Davis, 2013; Teranishi and Ueno, 2003). There is however no indication, at the time of writing, for any of the preceding mechanisms for glycogen phosphorylation.

Our own efforts to address the matter began with the proposed glucose-1-phosphotransferase. After synthesizing the needed [ $\beta$ - $^{32}$ P]UDP-glucose substrate, we showed that a mouse muscle extract could indeed transfer  $^{32}$ P to glycogen (Tagliabracci et

al., 2011), consistent with the hypothesis of Lomako et al. (Lomako et al., 1993). However, an extract from mouse muscle genetically lacking glycogen synthase was negative, suggesting that the phosphotransferase might be glycogen synthase itself. A series of *in vitro* experiments using highly purified glycogen synthase from rabbit muscle or purified recombinant glycogen synthase from yeast or from human confirmed that the enzyme mediated the introduction of phosphate when incubated with glycogen and UDP-glucose. We suggested that this was a rare side reaction happening at a rate of about one in 10,000 normal catalytic cycles. The  $^{32}\text{P}$  was reduced to background when the product was incubated either with glucosidase enzymes, to degrade the glycogen, or laforin, to hydrolyze phosphomonesters. How, then, might glycogen synthase catalyze the introduction of phosphate? One possibility was suggested by the early work of Leloir on nucleoside diphosphate sugars, notably UDP-glucose. His laboratory described its tendency, under certain conditions such as the presence of divalent metal cations, to form what he called “fast ester” because on TLC it ran ahead of UDP-glucose (Paladini and Leloir, 1952). Fast ester is, in fact, glucose-1,2-cyclic phosphate and we speculated that, if this diester could form in the catalytic site of glycogen synthase, it might be transferred by essentially the normal catalytic mechanism to a growing glycogen chain to add a glucose with phosphate at the C2 position (Tagliabracci et al., 2011). One could even envision a glucose-1,3-cyclic phosphate intermediate resulting in phosphate at C3. To prove such hypotheses is especially challenging but enzymological and structural studies did provide further support (Chikwana et al., 2013). First, a crystal structure of glycogen synthase with glucose-1,2-cyclic phosphate bound showed that the catalytic site could accommodate the cyclic ester in a manner consistent with the proposed mechanism. Secondly, incubation of glycogen synthase with UDP-glucose resulted in the generation of the cyclic phosphate.

This proposed mechanism for glycogen phosphorylation has been challenged by Nitschke et al. (Nitschke et al., 2013) who suggested that the  $^{32}\text{P}$ -incorporation into glycogen that we described (Tagliabracci et al., 2011) was not covalently attached phosphate but the result of tight binding to glycogen of the  $[\beta\text{-}^{32}\text{P}]\text{UDP}$  formed from  $[\beta\text{-}^{32}\text{P}]\text{UDP-glucose}$  by the normal glycogen synthase reaction. Our protocol for monitoring glycogen phosphorylation utilizes SDS-PAGE in which glycogen is mostly retained at the top of and within the stacking gel. Such binding to glycogen by UDP would need to be strong enough to survive the initial ethanol precipitation of glycogen, boiling in SDS gel electrophoresis buffer and the process of electrophoresis itself. Nitschke et al. (Nitschke et al., 2013) reported that the  $^{32}\text{P}$ -glycogen signal generated by such reactions was reduced to a low level by gel filtration with Sephadex G50 prior to SDS-PAGE, supporting their conclusion that the  $^{32}\text{P}$  was not covalently linked to glycogen. We have revisited our experiments and conducted several additional controls (Contreras, DePaoli-Roach and Roach, unpublished results). In our hands, gel filtration did not eliminate the  $^{32}\text{P}$  signal from glycogen analyzed by SDS-PAGE nor did addition of large molar excesses of unlabeled UDP. In addition, we confirmed that the  $^{32}\text{P}$ -labeling was removed from glycogen by laforin and furthermore showed that laforin was unable to hydrolyze the  $\beta$ -phosphate of UDP. Based on these, and other, results, we stand by our original conclusions regarding the phosphorylation of glycogen by glycogen synthase (Tagliabracci et al., 2011). It is very difficult to be certain of the explanations in instances of discordant results like this. We do indeed find that, at the

very high specific radioactivities needed for the experiments,  $^{32}\text{P}$  contamination of glycogen is an issue that needs to be carefully addressed. Our data, though, convince us that the  $^{32}\text{P}$ -signal that we monitor is neither non-specific radioactivity nor  $[\beta\text{-}^{32}\text{P}]\text{UDP}$ .

A glucose cyclic phosphate mechanism, such as described above, may account for the presence of C2 phosphate in glycogen, and perhaps even C3 phosphate since both glucose-1,2-cyclic phosphate and glucose-1,3-cyclic phosphate are known (Zmudzka and Shugar, 1964). Although C6 cyclic phosphates involving C3 or C4 have been described (Zmudzka and Shugar, 1964), a glucose-1,6-cyclic phosphate is sterically impossible, precluding its direct generation from UDP-glucose. It is hard to imagine how the  $\beta$ -phosphate of UDP-glucose, linked to C1 of the glucose, could end up at C6 unless via an intermediate glucose-3,6-cyclic phosphate, a rather complex mechanism. Therefore, the origin of the C6 phosphate remains a mystery and perhaps further exploration of some of the possibilities listed in Table 1, or another not thought of, will one day solve the puzzle.

## 2.4 Function

Normal glycogen contains a low, basal level of phosphorylation. As with any biological phenomenon, the first instinct is to seek a biological function for glycogen phosphate that can provide the evolutionary rationale for its existence. Is there a function for the trace amounts of covalent phosphate in glycogen? The question seems all the more relevant since there is a substantial experimental basis to suggest that phosphorylation of amylopectin, a biological cousin of glycogen, serves a definite purpose in the degradation of the polymer (Blennow et al., 2002; Fettke et al., 2009; Stitt and Zeeman, 2012; Zeeman et al., 2010). However, despite the considerable similarities in the overall metabolism and function of starch and glycogen, there are many key differences that make such comparative arguments less compelling. Unlike glycogen, starch contains highly structured, insoluble semi-crystalline segments not accessible to normal enzymatic attack (Blennow and Engelsen, 2010; Fettke et al., 2009; Perez and Bertoft, 2010). The role of phosphorylation in starch breakdown appears to be the disruption of that semi-crystalline structure, allowing exposure to water so that hydrolytic enzymes can access and cleave the polymer. A similar role in glycogen degradation seems unlikely. In fact, hyperphosphorylation of glycogen promotes insolubility in water (Tagliabracci et al., 2008). Could phosphorylation serve other evolved functions in glycogen? One suggestion has been that C6 phosphorylation is involved in determining  $\alpha$ -1,6-branches and thus might control the branching pattern of glycogen (Nitschke et al., 2013). Certainly a C6 phosphomonoester would block the ability to form an  $\alpha$ -1,6-glycosidic linkage but the frequency of C6 phosphorylation is very low so that less than 1% of potential branch points are affected (DePaoli-Roach et al., 2015). Even under conditions of pathologically elevated glycogen phosphorylation as in Lafora disease (Section 2.5), the frequency of C6 phosphorylation is increased no more than about 10-fold. There is clearly some relationship between excessive phosphorylation of glycogen and branching but any impact of phosphorylation on branching pattern is likely by a more indirect mechanism. Discovery of some glycogen phosphorylating activity (Table 1), one that requires additional enzyme(s) and/or protein(s), would provide powerful evidence that phosphorylation of glycogen serves an as yet undetermined purpose since maintenance of the associated genes would have had to withstand the selective pressures of evolution.



An alternative is that glycogen phosphorylation has no evolved function and is the result of a side reaction (Roach, 2011). For C2 and perhaps C3 phosphorylation, that side reaction could be mediated by glycogen synthase (Tagliabracci et al., 2011). Although many metabolic enzymes have apparently singular functions in cells, it is also clear that some enzymes can catalyze more than one chemical reaction, whether by design or by accident. In the interconversion of soluble small molecules, generation of trace amounts of physiologically irrelevant species by a side reaction or catalytic error may seldom have an impact. Such species may be excreted or metabolized by existing pathways. In the case of macromolecules, however, the situation is fundamentally different since most have significant lifetimes, some very long. The extreme is probably DNA which is synthesized once per cell division. And indeed, the severe consequence of errors in DNA synthesis has led to the evolution of complex mechanisms to ensure the fidelity of replication and to repair damage incurred post-synthesis (Kunkel and Bebenek, 2000). Similarly, sophisticated evolved mechanisms ensure the fidelity of RNA (Thomas et al., 1998) and protein synthesis (Zaher and Green, 2009). In glycogen, phosphate introduced by side reactions, or indeed by any mechanism, must remain attached to the polysaccharide through cycles of synthesis and degradation since there is normally a basal level of phosphorylation. However, excessive glycogen phosphorylation, such as when laforin is defective, has dire consequences and is to be avoided (Section 2.5). Laforin acts to oppose phosphate accumulation and thus might be viewed as part of a “repair” or “damage control” mechanism.

## 2.5 Lafora disease

Lafora disease (Andrade et al., 2007; Delgado-Escueta, 2007; Ganesh et al., 2006; Gentry et al., 2009; Roach and DePaoli-Roach, 2013) is a teenage-onset progressive myoclonic epilepsy that is inevitably fatal, usually within a decade of the appearance of symptoms. Approximately 90% of cases are caused by mutations in either of two genes, *EPM2A* and *EPM2B* (also called *NHLRC1*) (Ganesh et al., 2006; Ramachandran et al., 2009). As noted, *EPM2A* encodes laforin (Minassian et al., 1998; Serratos et al., 1999) and the *EPM2B* gene encodes malin which contains an N-terminal RING finger domain followed by six NHL domains (Chan et al., 2003). The RING finger domain is characteristic of E3 ubiquitin ligases (Freemont, 2000), an activity that has been demonstrated biochemically (Gentry et al., 2005). A characteristic of Lafora disease is the accumulation, in neurons, muscle, heart, skin and several other tissues, of Lafora bodies, insoluble deposits that contain polyglucosan, a poorly branched, glycogen-like polymer (Carpenter and Karpatis, 1981; Collins et al., 1968; DiMauro and Lamperti, 2001).

Since the identification of the laforin and malin genes, much effort has been directed at understanding the functions of the corresponding proteins. Most would agree that laforin can act as a physiological glycogen phosphatase (Tagliabracci et al., 2007). The function of malin is less clear. Based primarily on experiments with cultured cells, several malin substrates have been proposed (Cheng et al., 2007; Gentry et al., 2005; Moreno et al., 2010; Sharma et al., 2011; Solaz-Fuster et al., 2008; Vilchez et al., 2007; Worby et al., 2008) but results with *Epm2b*<sup>-/-</sup> mice (malin<sup>-/-</sup> mice) do not provide simple confirmation of these as malin targets (DePaoli-Roach et al., 2010). Nonetheless, in both patients and mice, defects in laforin and malin lead to generally similar phenotypes, both neurologically and

biochemically (Andrade et al., 2007; Delgado-Escueta, 2007; Ganesh et al., 2006; Garcia-Cabrero et al., 2012; Gentry et al., 2009; Roach and DePaoli-Roach, 2013). Both laforin<sup>-/-</sup> and malin<sup>-/-</sup> mice have elevated levels of glycogen and glycogen total phosphorylation (DePaoli-Roach et al., 2015; Tagliabracci et al., 2007; Turnbull et al., 2010). The fold increases in C6 and in C2 + C3 phosphate, in either laforin<sup>-/-</sup> or malin<sup>-/-</sup> mice, were similar so that, as of now, it is impossible to conclude that phosphorylation at any particular site in glucose is more important.

How does covalent phosphorylation of glycogen affect structure? Because the structure of glycogen is not amenable to determination by classic methods such as X-ray crystallography, it is difficult to probe directly the effect of the phosphate. However, the crystal structure of a cycloamylose, a 26-residue cyclic polymer of glucose in  $\alpha$ -1,4-linkages, has been determined (Gessler et al., 1999). The structure is composed of two antiparallel polyglucose helices with the glucose oxygens oriented toward the exterior surface and the carbon skeletons of the glucoses lining the interior of the helix. Although the helices are constrained by the cyclic nature of the oligosaccharide, it is likely that many features of the helices reflect those present in glycogen. In the cycloamylose structure, the helices are stabilized by intrahelical hydrogen bonding mediated by the physically adjacent C6, C2 and C3 hydroxyls. Therefore, introduction of phosphate groups to any of these glucose hydroxyl groups would disrupt the hydrogen bonding and potentially destabilize the structure. For amylopectin, it has been suggested that C3 phosphorylation induces strain in the helix; though C6 phosphate is more tolerated, it would also affect the packing of helices (Blennow and Engelsens, 2010).

These ideas are consistent with the notion that a relatively small number of phosphates in glycogen have far reaching effects on structure and on the physical chemical properties of the polymer. This is quite evident from the analysis of glycogen from laforin<sup>-/-</sup> mice (Tagliabracci et al., 2008). Muscle glycogen purified from these animals is more soluble in ethanol, to such a degree that we could genotype mice based solely on this property. The glycogen is less soluble in water and less branched. By electron microscopy, the glycogen from the laforin<sup>-/-</sup> mice forms large aggregates of individual glycogen particles that have a distinct appearance compared with wild type glycogen. Exposure to laforin returned the morphology of the laforin<sup>-/-</sup> glycogen closer to that of wild-type glycogen. Nonetheless, these major differences in the properties of glycogen were associated with only a ~10-fold increase in phosphorylation in the laforin<sup>-/-</sup> mice, from 1:1500 to 1:150 phosphates per glucose. The changes in glycogen properties in the absence of laforin, decreased water solubility and increased propensity to aggregate, would seem conducive to the formation of Lafora bodies. It should be emphasized that an important characteristic of the abnormal glycogen associated with Lafora bodies, besides elevated phosphate, is the reduced branching. The interplay between phosphorylation and branching structure in the generation of the abnormal glycogen of Lafora bodies remains an unresolved and critical issue in understanding the molecular basis of the disease.

Because of the abnormal metabolism of glycogen in the mouse models, one can ask whether Lafora disease should be classified as a glycogenosis. Several studies of genetically modified mice have indeed demonstrated that Lafora bodies are causative of the disease.



Both *laforin*<sup>-/-</sup> and *malin*<sup>-/-</sup> mice accumulate Lafora bodies and develop neurological abnormalities (Criado et al., 2012; DePaoli-Roach et al., 2015; DePaoli-Roach et al., 2010; Ganesh et al., 2002; Garcia-Cabrero et al., 2012; Tagliabracci et al., 2007; Turnbull et al., 2010; Valles-Ortega et al., 2011). PTG is a type 1 protein phosphatase targeting subunit that binds glycogen and activates glycogen synthase; *PTG*<sup>-/-</sup> mice have decreased glycogen accumulation in muscle and brain (Zhai et al., 2007). Disruption of the mouse *PTG* gene in either a *laforin*<sup>-/-</sup> (Turnbull et al., 2011) or *malin*<sup>-/-</sup> ((Turnbull et al., 2014); DePaoli-Roach, Segvich, Contreras and Roach, unpublished) genetic background decreases glycogen accumulation, suppresses Lafora body formation and alleviates neurological symptoms. In addition, complete or even partial elimination of brain glycogen by disruption of the *Gys1* gene in *laforin*<sup>-/-</sup> or *malin*<sup>-/-</sup> mice abolished Lafora body formation and restored neurological functions (Duran et al., 2014; Pederson et al., 2013). Thus, evidence is accumulating that Lafora disease is a glycogen storage disease and a promising therapeutic approach may be to suppress glycogen synthesis in the brain. Mice totally lacking brain glycogen, surprisingly, do not display any obvious neurological defects.

### 3. Conclusion

It is, I think, fair to say that we are quite far from understanding the phosphorylation of glycogen and its role in health and disease. However, it is fascinating to see how the field has progressed from the early report of Fontana (Fontana, 1980), through Bill Whelan's studies in the 1990s, to the recent work that was spurred on by the potential connection of glycogen phosphorylation with Lafora disease, a deadly neurological disorder. Bill Whelan, throughout his career, has had an uncanny ability to latch on to significant, sometimes seemingly simple, observations that end up being of considerable importance.

### Acknowledgments

Studies from the author's laboratory have been funded by NIH grants DK27221, NS056454 and HL108301

### References

- Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A, Godzik A, Hunter T, Dixon J, Mustelin T. Protein tyrosine phosphatases in the human genome. *Cell*. 2004; 117(6):699–711. [PubMed: 15186772]
- Andrade DM, Turnbull J, Minassian BA. Lafora disease, seizures and sugars. *Acta Myol*. 2007; 26(1): 83–86. [PubMed: 17915579]
- Baunsgaard L, Lutken H, Mikkelsen R, Glaring MA, Pham TT, Blennow A. A novel isoform of glucan, water dikinase phosphorylates pre-phosphorylated alpha-glucans and is involved in starch degradation in *Arabidopsis*. *Plant J*. 2005; 41(4):595–605. [PubMed: 15686522]
- Blennow A, Engelsens SB. Helix-breaking news: fighting crystalline starch energy deposits in the cell. *Trends in plant science*. 2010; 15(4):236–240. [PubMed: 20149714]
- Blennow A, Nielsen TH, Baunsgaard L, Mikkelsen R, Engelsens SB. Starch phosphorylation: a new front line in starch research. *Trends in plant science*. 2002; 7(10):445–450. [PubMed: 12399179]
- Carpenter S, Karpati G. Ultrastructural findings in Lafora disease. *Annals of neurology*. 1981; 10(1): 63–64. [PubMed: 6791573]
- Chan EM, Young EJ, Ianzano L, Munteanu I, Zhao X, Christopoulos CC, Avanzini G, Elia M, Ackerley CA, Jovic NJ, Bohlega S, Andermann E, Rouleau GA, Delgado-Escueta AV, Minassian

- BA, Scherer SW. Mutations in NHLRC1 cause progressive myoclonus epilepsy. *Nat Genet.* 2003; 35(2):125–127. [PubMed: 12958597]
- Cheng A, Zhang M, Gentry MS, Worby CA, Dixon JE, Saltiel AR. A role for AGL ubiquitination in the glycogen storage disorders of Lafora and Cori's disease. *Genes Dev.* 2007; 21(19):2399–2409. [PubMed: 17908927]
- Chikwana VM, Khanna M, Baskaran S, Tagliabracci VS, Contreras CJ, DePaoli-Roach AA, Roach PJ, Hurley TD. The structural basis of 2-phosphoglucose incorporation into glycogen by glycogen synthase. *Proc National Academy Sciences U.S.A.* 2013; 110:20976–20981.
- Collins GH, Cowden RR, Nevis AH. Myoclonus epilepsy with Lafora bodies. An ultrastructural and cytochemical study. *Arch Pathol.* 1968; 86(3):239–254. [PubMed: 4877594]
- Criado O, Aguado C, Gayarre J, Duran-Trio L, Garcia-Cabrero AM, Vernia S, San Millan B, Heredia M, Roma-Mateo C, Mouron S, Juana-Lopez L, Dominguez M, Navarro C, Serratosa JM, Sanchez M, Sanz P, Bovolenta P, Knecht E, Rodriguez de Cordoba S. Lafora bodies and neurological defects in malin-deficient mice correlate with impaired autophagy. *Hum Mol Genet.* 2012; 21(7):1521–1533. [PubMed: 22186026]
- Delgado-Escueta AV. Advances in lafora progressive myoclonus epilepsy. *Curr Neurol Neurosci Rep.* 2007; 7(5):428–433. [PubMed: 17764634]
- DePaoli-Roach AA, Contreras CJ, Segvich DM, Heiss C, Ishihara M, Azadi P, Roach PJ. Glycogen phosphomonoester distribution in mouse models of the progressive myoclonic epilepsy, Lafora disease. *J Biol Chem.* 2015; 290:841–850. [PubMed: 25416783]
- DePaoli-Roach AA, Tagliabracci VS, Segvich DM, Meyer CM, Irimia JM, Roach PJ. Genetic depletion of the malin E3 ubiquitin ligase in mice leads to lafora bodies and the accumulation of insoluble laforin. *J Biol Chem.* 2010; 285(33):25372–25381. [PubMed: 20538597]
- DiMauro S, Lamperti C. Muscle glycogenoses. *Muscle & Nerve.* 2001; 24(8):984–999. [PubMed: 11439374]
- Duran J, Gruart A, Garcia-Rocha M, Delgado-Garcia JM, Guinovart JJ. Glycogen accumulation underlies neurodegeneration and autophagy impairment in Lafora disease. *Hum Mol Genet.* 2014; 23(12):3147–3156. [PubMed: 24452334]
- Fettke J, Hejazi M, Smirnova J, Hochel E, Stage M, Steup M. Eukaryotic starch degradation: integration of plastidial and cytosolic pathways. *J Exp Bot.* 2009; 60(10):2907–2922. [PubMed: 19325165]
- Fontana JD. The presence of phosphate in glycogen. *FEBS Letters.* 1980; 109(1):85–92. [PubMed: 6153366]
- Freemont PS. RING for destruction? *Curr Biol.* 2000; 10(2):R84–87. [PubMed: 10662664]
- Fukuda T, Ewan L, Bauer M, Mattaliano RJ, Zaal K, Ralston E, Plotz PH, Raben N. Dysfunction of endocytic and autophagic pathways in a lysosomal storage disease. *Annals of neurology.* 2006; 59(4):700–708. [PubMed: 16532490]
- Ganesh S, Delgado-Escueta AV, Sakamoto T, Avila MR, Machado-Salas J, Hoshii Y, Akagi T, Gomi H, Suzuki T, Amano K, Agarwala KL, Hasegawa Y, Bai DS, Ishihara T, Hashikawa T, Itohara S, Cornford EM, Niki H, Yamakawa K. Targeted disruption of the Epm2a gene causes formation of Lafora inclusion bodies, neurodegeneration, ataxia, myoclonus epilepsy and impaired behavioral response in mice. *Human Molecular Genetics.* 2002; 11(11):1251–1262. [PubMed: 12019206]
- Ganesh S, Puri R, Singh S, Mittal S, Dubey D. Recent advances in the molecular basis of Lafora's progressive myoclonus epilepsy. *J Hum Genet.* 2006; 51(1):1–8. [PubMed: 16311711]
- Garcia-Cabrero AM, Marinas A, Guerrero R, de Cordoba SR, Serratosa JM, Sanchez MP. Laforin and malin deletions in mice produce similar neurologic impairments. *J Neuropathol Exp Neurol.* 2012; 71(5):413–421. [PubMed: 22487859]
- Gentry MS, Dixon JE, Worby CA. Lafora disease: insights into neurodegeneration from plant metabolism. *Trends Biochem Sci.* 2009; 34(12):628–639. [PubMed: 19818631]
- Gentry MS, Worby CA, Dixon JE. Insights into Lafora disease: malin is an E3 ubiquitin ligase that ubiquitinates and promotes the degradation of laforin. *Proc Natl Acad Sci U S A.* 2005; 102(24):8501–8506. [PubMed: 15930137]
- Gessler K, Uson I, Takaha T, Krauss N, Smith SM, Okada S, Sheldrick GM, Saenger W. V-Amylose at atomic resolution: X-ray structure of a cycloamylose with 26 glucose residues

- (cyclomaltohexaosaose). *Proc Natl Acad Sci U S A*. 1999; 96(8):4246–4251. [PubMed: 10200247]
- Hirschhorn, R.; Reuser, AJ. Glycogen Storage Disease Type II: Acid alpha-Glucosidase (Acid Maltase) Deficiency. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D., editors. *The Metabolic and Molecular Basis of Inherited Disease*. McGraw-Hill; New York: 2000. p. 3389–3420.
- Hizukuri S, Tabata S, Nikuni Z. Studies on starch phosphate. *Die Starke/Starch*. 1970; 22:338–343.
- Jiang S, Heller B, Tagliabracci VS, Zhai L, Irimia JM, DePaoli-Roach AA, Wells CD, Skurat AV, Roach PJ. Starch binding domain-containing protein 1/genethonin 1 is a novel participant in glycogen metabolism. *J Biol Chem*. 2010; 285(45):34960–34971. [PubMed: 20810658]
- Kennedy, LD.; Kirkman, BR.; Lomako, J.; Rodriguez, IR.; Whelan, WJ. The biogenesis of rabbit muscle glycogen. In: Berman, MC.; Gevers, W.; Opie, LH., editors. *Membranes and muscle*. ICSU Press/IRL Press; Oxford: 1985. p. 65–84.
- Kirkman BR, Whelan WJ. Intermolecular chemical heterogeneity of liver glycogen. *Biofactors*. 1990; 2(3):193–196. [PubMed: 2378672]
- Kotting O, Pusch K, Tiessen A, Geigenberger P, Steup M, Ritte G. Identification of a novel enzyme required for starch metabolism in Arabidopsis leaves. The phosphoglucan, water dikinase. *Plant Physiol*. 2005; 137(1):242–252. [PubMed: 15618411]
- Kunkel TA, Bebenek K. DNA replication fidelity. *Annu Rev Biochem*. 2000; 69:497–529. [PubMed: 10966467]
- Lomako J, Lomako WM, Kirkman BR, Whelan WJ. The role of phosphate in muscle glycogen. *Biofactors*. 1994; 4(3-4):167–171. [PubMed: 7916962]
- Lomako J, Lomako WM, Whelan WJ, Marchase RB. Glycogen contains phosphodiester groups that can be introduced by UDPglucose: glycogen glucose 1-phosphotransferase. *FEBS Letters*. 1993; 329(3):263–267. [PubMed: 8396041]
- MacDonald, DL. Phosphates and other inorganic esters, *The Carbohydrates 2E V1A*. Elsevier; 1972. p. 253–278.
- Mikkelsen R, Baunsgaard L, Blennow A. Functional characterization of alpha-glucan, water dikinase, the starch phosphorylating enzyme. *Biochem J*. 2004; 377(Pt 2):525–532. [PubMed: 14525539]
- Minassian BA, Lee JR, Herbrick JA, Huizenga J, Soder S, Mungall AJ, Dunham I, Gardner R, Fong CY, Carpenter S, Jardim L, Satishchandra P, Andermann E, Snead OC 3rd, Lopes-Cendes I, Tsui LC, Delgado-Escueta AV, Rouleau GA, Scherer SW. Mutations in a gene encoding a novel protein tyrosine phosphatase cause progressive myoclonus epilepsy. *Nature Genetics*. 1998; 20(2):171–174. [PubMed: 9771710]
- Mordoh J, Krisman CR, Leloir LF. Further studies on high molecular weight liver glycogen. *Arch Biochem Biophys*. 1966; 113(2):265–272. [PubMed: 5941189]
- Moreno D, Towler MC, Hardie DG, Knecht E, Sanz P. The laforin-malin complex, involved in Lafora disease, promotes the incorporation of K63-linked ubiquitin chains into AMP-activated protein kinase beta subunits. *Mol Biol Cell*. 2010; 21(15):2578–2588. [PubMed: 20534808]
- Nitschke F, Wang P, Schmieder P, Girard JM, Awrey DE, Wang T, Israelian J, Zhao X, Turnbull J, Heydenreich M, Kleinpeter E, Steup M, Minassian BA. Hyperphosphorylation of glucosyl C6 carbons and altered structure of glycogen in the neurodegenerative epilepsy Lafora disease. *Cell Metab*. 2013; 17(5):756–767. [PubMed: 23663739]
- Northcote DH. Electrophoresis of some neutral polysaccharides. *Biochem J*. 1954; 58(3):353–358. [PubMed: 13208620]
- Paladini AC, Leloir LF. Studies on uridine-diphosphate-glucose. *Biochem J*. 1952; 51(3):426–430. [PubMed: 12977745]
- Patel MK, Davis BG. Control of phosphoryl migratory transesterifications allows regioselective access to sugar phosphates. *Org Lett*. 2013; 15(2):346–349. [PubMed: 23286302]
- Pederson BA, Turnbull J, Epp JR, Weaver SA, Zhao X, Pencea N, Roach PJ, Frankland PW, Ackerley CA, Minassian BA. Inhibiting glycogen synthesis prevents lafora disease in a mouse model. *Annals of neurology*. 2013; 74(2):297–300. [PubMed: 23913475]
- Perez S, Bertoft E. The molecular structures of starch components and their contribution to the architecture of starch granules: A comprehensive review. *Starch-Stärke*. 2010; 62:389–420.

- Raben N, Plotz P, Byrne BJ. Acid alpha-glucosidase deficiency (glycogenosis type II, Pompe disease). *Curr Mol Med*. 2002; 2(2):145–166. [PubMed: 11949932]
- Ramachandran N, Girard JM, Turnbull J, Minassian BA. The autosomal recessively inherited progressive myoclonus epilepsies and their genes. *Epilepsia*. 2009; 50(Suppl 5):29–36. [PubMed: 19469843]
- Reuser AJ, Kroos MA, Hermans MM, Bijvoet AG, Verbeet MP, Van Diggelen OP, Kleijer WJ, Van der Ploeg AT. Glycogenosis type II (acid maltase deficiency). *Muscle Nerve*. 1995; 3:S61–69. [PubMed: 7603530]
- Roach PJ. Are there errors in glycogen biosynthesis and is laforin a repair enzyme? *FEBS Lett*. 2011; 585(20):3216–3218. [PubMed: 21930129]
- Roach, PJ.; DePaoli-Roach, AA. Glycogen metabolism and Lafora disease. In: Bence, KK., editor. *Protein tyrosine phosphatase control of metabolism*. Springer Science+Business Media; New York: 2013. p. 239-262.
- Serratos JM, Gomez-Garre P, Gallardo ME, Anta B, de Bernabe DB, Lindhout D, Augustijn PB, Tassinari CA, Malafosse RM, Topcu M, Grid D, Dravet C, Berkovic SF, de Cordoba SR. A novel protein tyrosine phosphatase gene is mutated in progressive myoclonus epilepsy of the Lafora type (EPM2). *Hum Mol Genet*. 1999; 8(2):345–352. [PubMed: 9931343]
- Sharma J, Rao SN, Shankar SK, Satishchandra P, Jana NR. Lafora disease ubiquitin ligase malin promotes proteasomal degradation of neuronatin and regulates glycogen synthesis. *Neurobiol Dis*. 2011; 44(1):133–141. [PubMed: 21742036]
- Solaz-Fuster MC, Gimeno-Alcaniz JV, Ros S, Fernandez-Sanchez ME, Garcia-Fojeda B, Criado Garcia O, Vilchez D, Dominguez J, Garcia-Rocha M, Sanchez-Piris M, Aguado C, Knecht E, Serratos J, Guinovart JJ, Sanz P, Rodriguez de Cordoba S. Regulation of glycogen synthesis by the laforin-malin complex is modulated by the AMP-activated protein kinase pathway. *Hum Mol Genet*. 2008; 17(5):667–678. [PubMed: 18029386]
- Stitt M, Zeeman SC. Starch turnover: pathways, regulation and role in growth. *Current opinion in plant biology*. 2012; 15(3):282–292. [PubMed: 22541711]
- Tagliabracci VS, Girard JM, Segvich D, Meyer C, Turnbull J, Zhao X, Minassian BA, Depaoli-Roach AA, Roach PJ. Abnormal metabolism of glycogen phosphate as a cause for Lafora disease. *J Biol Chem*. 2008; 283(49):33816–33825. [PubMed: 18852261]
- Tagliabracci VS, Heiss C, Karthik C, Contreras CJ, Glushka J, Ishihara M, Azadi P, Hurley TD, DePaoli-Roach AA, Roach PJ. Phosphate incorporation during glycogen synthesis and Lafora disease. *Cell Metab*. 2011; 13(3):274–282. [PubMed: 21356517]
- Tagliabracci VS, Turnbull J, Wang W, Girard JM, Zhao X, Skurat AV, Delgado-Escueta AV, Minassian BA, Depaoli-Roach AA, Roach PJ. Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen in vivo. *Proc Natl Acad Sci U S A*. 2007; 104(49):19262–19266. [PubMed: 18040046]
- Teranishi K, Ueno F. Mechanism of 2-O - 3-O silyl migration in cyclomaltosehexaose (a-cyclodextrin). *Tetrahedron Letters*. 2003; 44:4843–4848.
- Thomas MJ, Platas AA, Hawley DK. Transcriptional fidelity and proofreading by RNA polymerase II. *Cell*. 1998; 93(4):627–637. [PubMed: 9604937]
- Turnbull J, DePaoli-Roach AA, Zhao X, Cortez MA, Pencea N, Tiberia E, Piliguian M, Roach PJ, Wang P, Ackerley CA, Minassian BA. PTG depletion removes Lafora bodies and rescues the fatal epilepsy of Lafora disease. *PLoS Genet*. 2011; 7(4):e1002037. [PubMed: 21552327]
- Turnbull J, Epp JR, Goldsmith D, Zhao X, Pencea N, Wang P, Frankland PW, Ackerley CA, Minassian BA. PTG protein depletion rescues malin-deficient Lafora disease in mouse. *Annals of neurology*. 2014; 75(3):442–446. [PubMed: 24419970]
- Turnbull J, Wang P, Girard JM, Ruggieri A, Wang TJ, Draginov AG, Kameka AP, Pencea N, Zhao X, Ackerley CA, Minassian BA. Glycogen hyperphosphorylation underlies lafora body formation. *Annals of neurology*. 2010; 68(6):925–933. [PubMed: 21077101]
- Valles-Ortega J, Duran J, Garcia-Rocha M, Bosch C, Saez I, Pujadas L, Serafin A, Canas X, Soriano E, Delgado-Garcia JM, Gruart A, Guinovart JJ. Neurodegeneration and functional impairments associated with glycogen synthase accumulation in a mouse model of Lafora disease. *EMBO Mol Med*. 2011; 3(11):667–681. [PubMed: 21882344]

- Vilchez D, Ros S, Cifuentes D, Pujadas L, Valles J, Garcia-Fojeda B, Criado-Garcia O, Fernandez-Sanchez E, Medrano-Fernandez I, Dominguez J, Garcia-Rocha M, Soriano E, Rodriguez de Cordoba S, Guinovart JJ. Mechanism suppressing glycogen synthesis in neurons and its demise in progressive myoclonus epilepsy. *Nat Neurosci.* 2007; 10(11):1407–1413. [PubMed: 17952067]
- Wanson JC, Drochmans P. Rabbit skeletal muscle glycogen. A morphological and biochemical study of glycogen beta-particles isolated by the precipitation-centrifugation method. *The Journal of cell biology.* 1968; 38(1):130–150. [PubMed: 5691969]
- Worby CA, Gentry MS, Dixon JE. Laforin: A dual specificity phosphatase that dephosphorylates complex carbohydrates. *J Biol Chem.* 2006; 281:30412–30418. [PubMed: 16901901]
- Worby CA, Gentry MS, Dixon JE. Malin decreases glycogen accumulation by promoting the degradation of protein targeting to glycogen (PTG). *J Biol Chem.* 2008; 283(7):4069–4076. [PubMed: 18070875]
- Zaher HS, Green R. Fidelity at the molecular level: lessons from protein synthesis. *Cell.* 2009; 136(4):746–762. [PubMed: 19239893]
- Zeeman SC, Kossmann J, Smith AM. Starch: its metabolism, evolution, and biotechnological modification in plants. *Annual review of plant biology.* 2010; 61:209–234.
- Zhai L, Choi CS, Irimia-Dominguez J, McGuire AC, Kim S, Bock CB, Roach PJ, Shulman GI, DePaoli-Roach AA. Enhanced insulin sensitivity and energy expenditure in PPP1R3C (PTG) deleted mice. *Diabetes.* 2007; 56(Supplement 1):A62.
- Zmudzka BS, Shugar D. Preparation and chemical and enzymic properties of cyclic phosphates of D-glucopyranose and synthesis of derivatives of N-(D-glucopyranosyl) pyridine. *Acta Biochimica Polonica.* 1964; 11:509–525.

**Table 1**  
**Some possible mechanisms to mediate glycogen phosphorylation**

Shown are some potential enzymic reactions that could introduce phosphate into glycogen or re-arrange its location. X~P, unspecified high energy phosphate donor.

| Enzyme                       | Reaction  | Group transferred   |
|------------------------------|---|---------------------|
| Glucose-1-phosphotransferase | $\text{UDP-glucose} + \text{glycogen}_n \rightarrow \text{UMP} + \text{glycogen}_{n+1}\text{-P}$            | glucose-1-phosphate |
| Glycogen synthase            | $\text{UDP-glucose} + \text{glycogen}_n \rightarrow \text{UMP} + \text{glycogen}_{n+1}\text{-P}$            | glucose-1-phosphate |
| Glycogen dikinase            | $\text{ATP} + \text{H}_2\text{O} + \text{glycogen} \rightarrow \text{AMP} + \text{P}_i + \text{glycogen-P}$ | $\text{P}_i$        |
| Glycogen kinase              | $\text{NTP} + \text{glycogen} \rightarrow \text{NDP} + \text{glycogen-P}$                                   | $\text{P}_i$        |
| Other phosphotransferase     | $\text{X~P} + \text{glycogen} \rightarrow \text{X} + \text{glycogen-P}$                                     | $\text{P}_i$        |
| Mutase                       | $\text{glycogen-P}_{(\text{site } 1)} \rightarrow \text{glycogen-P}_{(\text{site } 2)}$                     | $\text{P}_i$        |